PHOSPHATE TRANSPORT IN RAT LIVER MITOCHONDRIA EVIDENCES FOR AN ENERGY LINKED P_i MEMBRANE BINDING

Martine GUERIN and Bernard GUERIN
Laboratoire de Biochimie, U.E.R. de Biochimie Biologie Cellulaire.
Université de Bordeaux II, 351, cours de la Libération, 33405 Talence, France

Received 11 December 1974

1. Introduction

The phosphate transport through the inner mitochondrial membrane has been found to be inhibited by thiol reagents [1,2]. We have reported previously that this inhibition was different with regard to two kinds of SH-reagent: maleimides, and Ellman's reagent were partial or total inhibitors according to the experimental conditions, however, mersalyl completely and consistently blocked phosphate transport, no matter the conditions applied [3]. From this observation and the analysis of the reactivity and permeability of some maleimides, it was proposed that P_i-carrier could be regarded as a mobile or reorientating carrier [3,4]. We found earlier that phosphate was able to protect its carrier from weak SH-reagent, but not from mersalyl [4,5]. This effect seems due mainly to an orientation of SH-groups of the carrier towards the inner side, according to the observation that internal phosphate is more effective than the external one [4].

It is shown in this communication that mersalyl removes phosphate when added after a phosphate accumulation is achieved (see also ref. [6]). This observation is discussed in relation to the hypothesis of the mobile or reorientating carrier.

2. Materials and methods

Rat liver mitochondria (Wistar) were isolated as described by Klingenberg and Slencza [7]. Then they were depleted of endogenous P_i as follows: mitochondria (100 mg protein) were suspended in 30 ml of a medium containing 20 mM Tris, 145 mM KCl, 2 mM $MgCl_2$, pH 7.4 and incubated for 5 min at 20°C with 300 mg glucose and 3 units hexokinase (Sigma, type III). After two washings in the same medium, mitochondria were resuspended in a 0.25 M sucrose, 1 mM EGTA, 10 mM Tris–maleate buffer, pH 6.6. The ^{32}P-phosphate uptake experiments were performed at 4°C on 0.4 ml of this suspension containing 2 mg protein and 4 μg oligomycin (for other additions see the text). After a rapid centrifugation, the pellet was suspended in 0.2 ml of water, deproteinized, centrifuged and the supernatant was routinely counted, utilizing the Cerenkof effect [8]. In some experiments the inorganic phosphate was separated from the extract [9] before the liquid scintillation counting. (12 ml of a toluene solution, 0.4% PPO, 0.01% POPOP + 3 ml of ethanol).

^{32}P-phosphate was obtained from C.E.A., Saclay (France). Ethylene glycol-bis (β amino-ethyl ether) *N-N'*-tetraacetate (EGTA), carbonyl cyanide *m*-chloro-phenyl hydrazone (CCP), valinomycin, antimycin A, oligomycin and mersalyl were purchased from Sigma.

3. Results

In order to prevent the dilution of the ^{32}P-phosphate by the internal phosphate, mitochondria were depleted as described under methods. Then, mitochondria were incubated with ^{32}P-phosphate for 2 min (see under fig.1). It was verified that after this interval no further P_i incorporation occurred. Some samples were centrifuged and the radioactivity counted in the

 North-Holland Publishing Company – Amsterdam

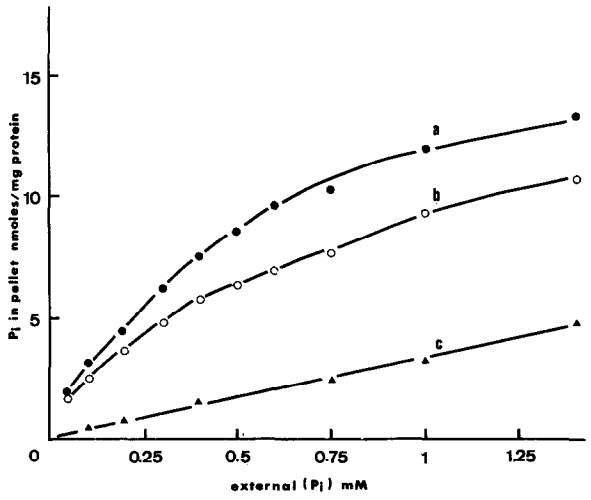

Fig.1. Effect of mersalyl on ^{32}P-phosphate uptake by rat liver mitochondria. Dependence of the external phosphate concentration. P_i-depleted mitochondria (2 mg protein) were incubated 1 min at 4°C in 0.4 ml of the following medium: 0.25 M sucrose, 1 mM EGTA, 10 mM Tris–maleate pH 6.6, oligomycin 4 μg. Expt. a: ^{32}P-phosphate was added and mitochondria were centrifuged 2 min after, (●—●—●). Expt. b: mitochondria were treated as for Expt. a, but mersalyl (50 nmol) was added 2 min after ^{32}P-phosphate, (○—○—○). Expt. c: mersalyl was added 1 min before ^{32}P-phosphate, (▲—▲—▲).

pellet. In other samples, mersalyl was added at a concentration inhibiting the phosphate transport; after 1 min, mitochondria were centrifuged and the pellet counted as above. The difference of the incorporation between the two samples corresponds to the amount of phosphate which was removed by mersalyl (Δ P_i). Fig.1 shows the results of these experiments as a function of external phosphate concentration (curves a and b). It was shown that mersalyl removed a significant amount of phosphate. This Δ P_i increased as a function of the phosphate concentration and reached a maximum value of 2 to 3 nmol/mg protein^{-1}. When mersalyl was added before phosphate, the transport was inhibited (curve c). In this case, the observed radioactivity followed a linear increase with added P_i and was localized only in the sucrose per-

Table 1
Effect of uncoupler, ionophores and respiratory inhibitors on the extent of phosphate uptake and Δ phosphate

	Addition	Phosphate uptake nmol/mg protein		Δ phosphate nmol/mg protein	
		P_i	P_o	Δ P_i	Δ P_o
Expt. 1	none	13.8	1	−1.8	< 0.2
	CCP	3.2	< 0.2	+0.4	< 0.2
	CCP + valinomycin	0.5	< 0.2	0.2	< 0.2
	nigericin	0.5	< 0.2	+0.4	< 0.2
Expt. 2	none	16.5	1.8	−1.5	< 0.2
	rotenone	13.1	0.6	<0.2	< 0.2
	rotenone + succinate	15.4	1.1	−2.1	< 0.2
	rotenone + antimycin + succinate	12.5	0.4	+0.3	< 0.2
	β hydroxybutyrate	15.8	1.1	−2.3	< 0.2
	rotenone + β hydroxybutyrate	12.8	0.7	<0.2	< 0.2
	butylmalonate	15.6	1.6	−1.3	< 0.2

Conditions and technique for measuring phosphate uptake and Δ phosphate were as described in fig.1. Organic (P_o) and inorganic (P_i) phosphate were separated before counting [9]. External P_i: 1 mM, mersalyl: 25 nmol per mg protein. As indicated, the additions were 1 μM CCP, 0.02 μg valinomycin, 0.02 μg nigericin, 2 μg rotenone, 2.5 μg antimycin, 1 mM β hydroxybutyrate, 1 mM succinate, 5 mM butylmalonate.

meable space (not shown). In a series of experiments the inorganic ^{32}P-phosphate was separated from the extract before counting. It is shown on table 1 that the phosphate removed by mersalyl was only inorganic.

We measured simultaneously and under the same conditions the phosphate transport inhibition by mersalyl and the amount of phosphate removed by this mersalyl concentration. The phosphate transport inhibition was measured first by mitochondrial swelling in a 100 mM ammonium phosphate buffer, and secondly by ^{32}P-phosphate incorporation; in both cases mitochondria were preincubated for 1 min with different mersalyl concentrations. The two curves were in agreement; a full inhibition of the P_i uptake was reached for 15 nm/mg protein (fig.2). When mersalyl was added after P_i uptake is achieved,

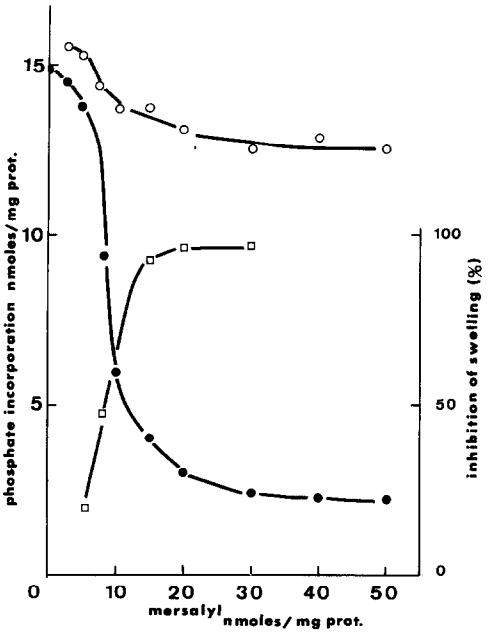

Fig.2. Titration of the effect of mersalyl on the phosphate uptake and on the ΔP_i. Mitochondria were incubated with different amounts of mersalyl. Swelling in 0.1 M ammonium phosphate, pH 7.2, was monitored in an Eppendorf photometer at 546 nm; the inhibition (%) of the rate of swelling was estimated, (□—□—□). Mitochondria were incubated as in fig.1, but at a constant phosphate concentration (1 mM) and different amounts of mersalyl as indicated in the figure. (●—●—●), mersalyl added before ^{32}P-phosphate. (○—○—○), mersalyl added 2 min after ^{32}P-phosphate.

the ΔP_i observed increased as a function of mersalyl concentration and reached a maximum value when 100% inhibition of phosphate transport was obtained (fig.2). It should be noted that this ΔP_i was not abolished by a concentration of butylmalonate sufficient to inhibit the P_i-dicarboxylate exchange [10] (table 1) and therefore did not represent an efflux of endogenous phosphate via the dicarboxylate carrier.

It is known that the net uptake of phosphate depends on the trans membrane Δ pH, [11]. In order to elucidate the role of accumulated P_i on the ΔP_i, the influence of ionophores and uncouplers was studied. The results are summarized in table 1. The uncoupler CCP, known to permeabilize the membrane to H^+, decreased the P_i uptake level and abolished the ΔP_i. Nigericin or valinomycin + CCP that catalysed an H^+/K^+ exchange, abolished the Δ pH and consequently the accumulation of P_i [12]; in these cases no ΔP_i was observed.

The effect of respiratory substrates and inhibitors on the ΔP_i were also studied (table 1). Although the P_i incorporation was decreased in the presence of rotenone, an important uptake was observed probably at the expense of the preexisting pH gradient; however no ΔP_i was obtained in these conditions. It is shown also that succinate, but not β-hydroxybutyrate, is able to restore the ΔP_i and that this effect is abolished by antimycin.

4. Discussion

It is well known that organic mercurial compounds inhibit the P_i transport at levels low enough that do not affect other mitochondrial functions [1,2]. Therefore, the titration curve of the phosphate removing effect of mersalyl strongly suggests that the ΔP_i is a consequence of the P_i-carrier inhibition. Although, mersalyl (a polar mercurial) seems to react only with the external thiol-groups, as in the case of *p*-chloromercuriphenyl sulfonate [13,14], it inhibits completely the influx and efflux of phosphate [3]. This observation can be explained in two ways: 1) the thiol-groups of the carrier are located on the outside of internal membrane: this interpretation is ruled out by a great number of data [3,4]; 2) the P_i-carrier can be regarded as a mobile or reorientating carrier

distributed between two positions (or conformations) at both sides of the inner mitochondrial membrane; consequently, the mersalyl must induce a reorientation of the carrier, from the internal towards the external position, leading also to other structural changes.

An explanation of the ΔP_i observed can be deduced from the effect of mersalyl on P_i-carrier. From the hypothesis of a mobile or reorientating carrier, the distribution of this one between both localisations depends mainly of the P_i concentration ratio between the two compartments. At the equilibrium (when the P_i accumulation is accomplished) internal concentration of P_i is higher than the external one. It can be deduced that: 1) the carrier is quantitatively directed toward the matrix; 2) a largest portion of the carrier (and other mitochondrial components) directed towards the inner side are loaded with P_i compared to the one directed towards the outer side. Consequently, in inducing conformational changes, mersalyl modifies the equilibrium between free and loaded P_i binding sites and phosphate is removed to the external medium.

This mechanism could explain why CCP, CCP + valinomycin and nigericin that decrease the ratio P_i int/P_i ext abolish ΔP_i [12]. However, a different situation is obtained with respiratory inhibitors. They decrease only slightly the uptake of phosphate at concentrations which inhibit oxidative phosphorylation but abolish the ΔP_i. These observations may reflect a more complex situation in which the ΔP_i is related to an energy linked conformation of the membrane and to the level of P_i uptake. It should be noted that the conformational change of the P_i carrier by mersalyl is probably accompanied by other structural modifications in the internal membrane and that the number of P_i binding sites at the carrier level may be less than two nmol per mg protein.

Acknowledgements

This work was supported by research grants from the Délégation Générale à la Recherche Scientifique et Technique and from the Fondation pour la Recherche Médicale.

References

[1] Fonyo, A. (1968) Biochem. Biophys. Res. Commun. 32, 624–628.
[2] Tyler, D. (1969) Biochem. J. 111, 665–668.
[3] Guerin, B., Guerin, M. and Klingenberg, M. (1970) FEBS Lett. 10, 265–268.
[4] Klingenberg, M., Durand, R. and Guerin, B. (1974) Eur. J. Biochem. 42, 135–150.
[5] Guerin, M. (1971) Thèse de Doctorat d'Etat, Université de Paris XI.
[6] Guerin, M. and Guerin, B. (1972) Abstr. Commun. 8th Meet. Fed. Eur. Biochem. Soc. 173.
[7] Klingenberg, M. and Slenczka, W. (1959) Biochem. Z. 331, 486–495.
[8] Clausen, T. (1969) Anal. Biochem. 22, 70–73.
[9] Heldt, H. W., Klingenberg, M. and Milovancev, M. (1972) Eur. J. Biochem. 30, 434–440.
[10] Robinson, B. H. and Williams, G. R. (1970) Biochim. Biophys. Acta 216, 63–70.
[11] Palmieri, F., Quagliariello, E. and Klingenberg, M. (1970) Eur. J. Biochem. 17, 230–238.
[12] Quagliariello, E. and Palmieri, F. (1970) FEBS Lett. 8, 105–108.
[13] Scott, K. M., Knight, V. A., Settlemire, C. T. and Brierly, G. P. (1970) Biochemistry 9, 714–723.
[14] Brierly, G. P. (1974) Ann. N.Y. Acad. Sci. 227, 398–411.